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Proteomics of the Circadian Clock

Franziska Brüning

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Erklärung

Diese Dissertation wurde im Sinne von §7 der Promotionsordnung vom 28. November 2011 von Frau Prof. Dr. Maria S. Robles betreut und von Herrn Prof. Dr. Matthias Mann von der Fakultät für Chemie und Pharmazie vertreten.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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Franziska Brüning

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Für meine Großeltern,
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Summary

The circadian clock is an internal time keeping system evolved to anticipate and adapt the physiology of organisms to recurring daily environmental changes. In mammals, the circadian system has a hierarchical structure with a master clock in the suprachiasmatic nucleus (SCN) of the brain. This master clock entrains self-autonomous clocks in peripheral organs which drive tissue-specific outputs to thus control local metabolism.
In every tissue (including the SCN) and in each cell throughout the body, the molecular mechanism of the circadian clock is based on transcriptional-translational feedback loops. At the core of the positive limb are the transcription factors BMAL1 and CLOCK, which drive the transcription of many genes including their own repressors CRYs and PERs. Many additional proteins have been identified to bind core clock proteins and thus regulate circadian gene expression. However, until now only fragments of this highly complex interaction network are known. For example, it is still not fully understood how exactly the protein composition of the core clock complex at the chromatin changes over time and how potentially the "same" molecular machinery regulates distinct rhythmic gene expression in different tissues to adapt local metabolism to external cues. Thus, one aim of this thesis was to establish a mass spectrometry (MS) based method to study the DNA-bound BMAL1:CLOCK complex in a time- and tissue-dependent manner. I successfully optimized a chromatin immunoprecipitation (ChIP) method followed by MS to purify endogenous complexes in human cell lines and murine tissues that contain the core clock components CLOCK and BMAL1. In this way I obtained promising preliminary results by finding already known core clock components and many potential new interactors.
One important physiological output of the circadian clock is the fundamental process of sleep which is regulated in part by circadian factors and also by homeostatic factors. The molecular interaction of these two pathways in fine tuning the control of sleep remains still unclear. Thus, another aim of my thesis was to dissect the influence of the circadian clock and the sleep homeostat on the synaptic molecular network and to investigate how this network is affected by the lack of sleep or sleep deprivation. To do that, in collaboration with Sara Noya and Steven Brown (University of Zurich), we studied daily oscillations of transcripts, proteins and phosphorylation in total forebrain and isolated synapses from well-rested as well as sleep-deprived mice. Our multi-omics study allowed us to identify the contribution of the circadian clock and sleep-wake mechanisms in the daily dynamics of synaptic processes. In brief, we found that across the day many transcripts are transported to the synapses where they are locally translated giving rise to cycles of protein abundance. Interestingly, sleep-deprivation does not affect the transport of transcripts and thus their abundance in synapses, indicating that this process is largely regulated by the circadian clock. However, local translation and thus cycles of protein abundances are completely abolished under sleep-

deprived conditions.
Moreover, our phosphoproteome study, quantifying more than 7000 phosphopeptides in synaptic proteins, showed an extensive degree of daily phosphorylation rhythms (30%). These rhythms were independent of protein oscillations, suggesting an important role of phosphorylation regulating distinct time of day processes at synapses. Remarkably, the phase distribution of these oscillating phosphopeptides revealed two major clusters, corresponding to dawn and dusk or to the transition from wake to sleep and vice versa. Among the quantified cycling phosphopeptides, a variety originated from kinases from all major kinase families indicating that phospho-dependent activation of kinases in response to rest-activity cycles is a key driver to regulate synaptic homeostasis and function.
To distinguish whether these oscillations are driven by the circadian clock or the sleep homeostat, we compared rhythmic phosphorylation of well-rested and sleep-deprived mice and found that, remarkable, 98% of rhythmic phosphorylations were lost due to sleep-deprivation. We thus concluded that sleep-wake cycles are the main driver of daily phosphorylation events at synapses. Together, our studies represent the first comprehensive overview of daily synaptic transcript, protein and phosphorylation cycles and provides experimental evidences that sleep need and synaptic function are tightly linked.

Contents

List of Abbreviations

2D-DIGE	2-dimensional-difference gel electrophoresis
AP-MS	Affinity purification mass spectrometry
bHLH	Basic helix-loop-helix
CCGs	Clock controlled genes
ChIP-MS	Chromatin immunoprecipation followed by mass spectrometry
EEG	Electroencephalogram
ESI	Electrospray ionization
FDR	False discovery rate
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HPLC	High performance liquid chromatography
iBAQ	Intensity-based absolute quantification
iTRAQ	Isobaric tags for relative and absolute quantitation
LFQ	Label-free quantification
MS	Mass spectrometry
PAS	Period-Arnt-Single-minded
PTM	Post-translational modification
REM	Rapid eye movement
SCN	Suprachiasmatic nucleus
SHY	Synaptic homeostasis hypothesis
SILAC	Stable isotope labeling by amino acids in cell culture
SWA	Slow wave activity
TMT	Tandem mass tag
ZT	Zeitgeber time

1 Introduction

1.1 Circadian Clock

The circadian clock is an internal time keeping system that is highly conserved among different species. The word "circadian" originates from the Latin *"circa diem"* that means "about a day". This is due to the fact that this timing system has evolved to anticipate and adapt the physiology of organisms to recurring daily environmental changes, such as light and dark conditions, due to the rotation of the earth [1]. The circadian clock runs thus in a self-autonomous manner with a periodicity of approximately 24h but can synchronize to environmental signals, termed "Zeitgeber". The adaptation of the internal body clock to those environmental cues is called entrainment. Light is one of the most important Zeitgebers and organisms naturally entrain to it through advancing or delaying the circadian clock [2, 3].
In mammals, the circadian system has a hierarchical structure with a master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus which receives light information from the retina ganglion cells [4, 5]. This information is then sent via systemic signals including neural, humoral and temperature to the peripheral organs which harbour their own self-autonomous clocks [6–9]. These signals are essential for all organs to run in synchrony [10]. In addition to light, the peripheral clocks synchronize to other Zeitgebers, for instance food intake entrains the liver clock, acting as an additional level of local control in peripheral tissues, which highlights the complex regulation of circadian rhythms *in vivo* [11, 12].
In modern society, mistimed exposure to Zeitgebers, for example being exposed to artificial light or consume food at night, can lead to adverse health outcome, such as obesity and cancer, due to the disruption of the circadian homoeostasis. Understanding the interplay between the different body clocks and the molecular mechanisms behind may allow to improve the health of people with high risks e.g. pilots and nurses, which routinely suffer circadian desynchronization due to their profession [2].

1.1.1 Molecular Mechanism

In every tissue (including the SCN) and in each cell throughout the body, the molecular mechanism of the circadian clock is based on transcriptional-translational feedback loops formed by a group of highly conserved proteins [reviewed in [13]].
In mammals, two transcription factors belonging to the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) family, BMAL1 (also called ARNTL) and CLOCK, are at the core of the positive limb of the feedback loops. As a heterodimer they bind to consensus motifs (E-boxes) in promoters of target genes to drive their transcription. Among these clock controlled genes (CCGs) are their own repressors *Period* (*Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) [14–18].

According to present knowledge and in a very simplistic way, PERs and CRYs form a complex in the cytoplasm and translocate to the nucleus to repress BMAL1:CLOCK transcriptional activity and thus their own transcription, thereby closing the loop which takes approximately 24h [17, 19].
In addition to this primary transcriptional-translational feedback loop, there is another loop regulating *Bmal1* transcription. It consists of the retinoic acid-related orphan nuclear receptors Rev-erbα and Rorα, which are also transcriptional targets of BMAL1:CLOCK. Both, Rev-erbα and Rorα bind to orphan receptor response elements located in the promoter sequence of *Bmal1*. While RORs (ROR α, β and γ) positively regulate its transcription, REV-ERBs (REV-ERB α and β) repress it, hence influencing circadian output oscillations [20–23]. The described auto-regulatory feedback loops, driving the circadian output signals, are illustrated in Fig. 1.

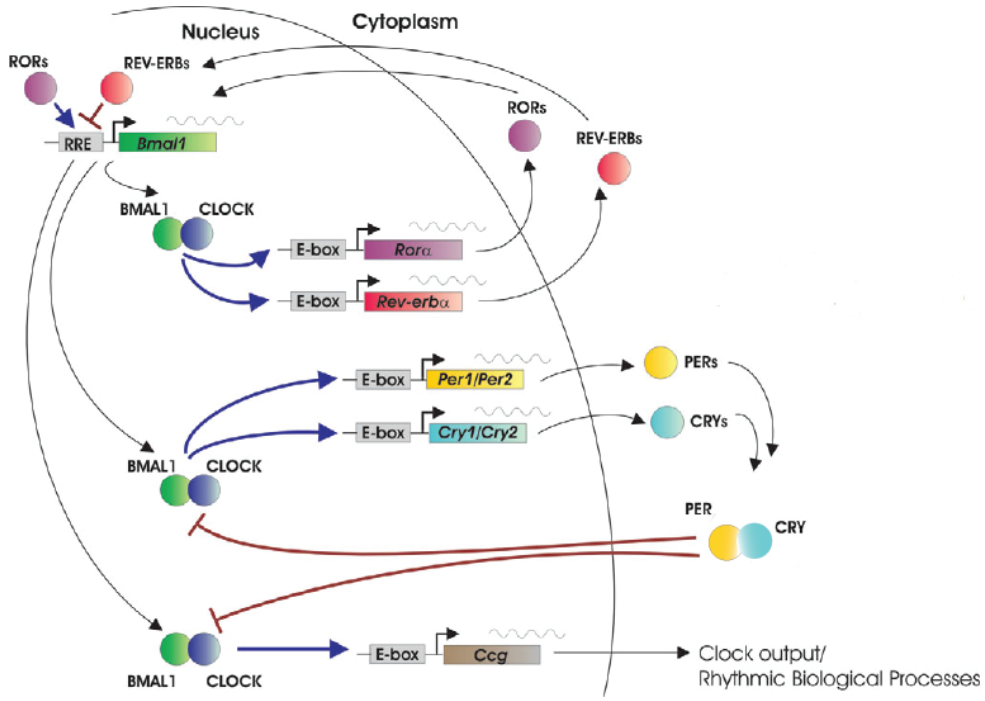

Fig. 1. Transcriptional-translational auto-regulatory feedback loops that drive circadian oscillations in mammals. Adapted from [24].

The timing of these loops is shaped by post-translational modifications (PTMs) on core clock proteins such as phosphorylation, acetylation, SUMOylation and ubiquitination. These PTMs regulate various mechanisms for example protein stability, translocation in the nucleus, interaction and binding to DNA [reviewed in [25]]. Moreover, some of these PTMs lead also to changes of the chromatin state. For instance, CLOCK has been shown to have a histone acetyltransferase (HAT) activity which is enhanced by its interaction with BMAL1 [26]. The interaction with BMAL1 is also suggested to lead to the recruitment of HAT cofactors to ultimately remodel the chromatin for transcription. Additionally, the

recruitment of other co-activators, such as members of the transcriptional mediator complex and RNA Polymerase II, to BMAL1:CLOCK seems to enable transcription of clock genes such as PERs, CRYs and others (Fig. 2). This finding is in line with the study of Trott and Menet, which proposes that BMAL1:CLOCK regulates transcription not by acting as transcription factors but rather by rhythmically remodelling the chromatin, allowing other transcription factors to access their promoters [27].

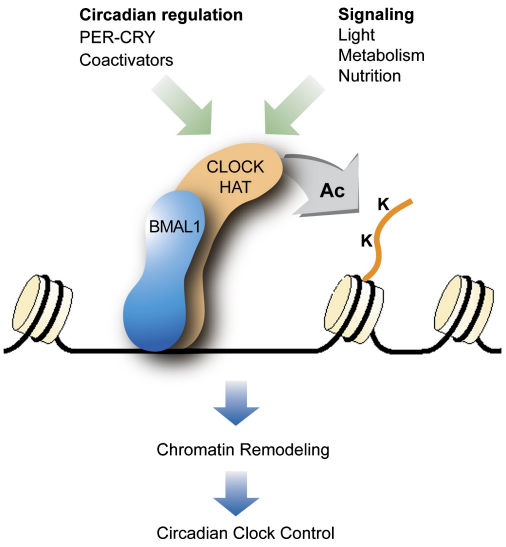

Fig. 2. Proposed model how the histone acetyltransferase activity of CLOCK is thought to modify the chromatin to drive rhythmic gene transcription. Adapted from [26].

The binding of repressors to the BMAL1:CLOCK complex at the chromatin is, in turn, associated, among others, with the recruitment of deacetylases, thereby generating a chromatin inhibitory state promoting transcriptional repression and thus helping to close the transcriptional-translational feedback loop [28–30].

An example of another PTM with great influence on the molecular core clock machinery is phosphorylation. Several kinases in particular kinase 1 epsilon (CK1ϵ) and Casein kinase 1 delta (CK1δ) phosphorylate core clock proteins such as the PER proteins thereby affecting e.g. protein stability and localization [31, 32]. Consequently, various regulatory mechanism including PTMs and selective protein interactions are necessary to optimally tune the timing of the transcriptional-translational feedback loops.

Despite of its importance, it is still not well understood how the interaction network of core clock proteins exactly looks like. Moreover, there are still pieces missing to define in detail how transcriptional, translational as well as post-translational mechanism work together to shape circadian metabolism. Studying interaction networks of core clock proteins in different tissues might thus shed light on how the clock drives local physiology.

1.1.2 Circadian Outputs

1.1.2.1 Tissue-specific Metabolism

Peripheral organs perform a variety of different task in the body and their clocks have to respond to the synchronization signals from the SCN; hence the tissue clock drives tissue-specific outputs to control local metabolism. For instance the clocks in liver and pancreas are involved in glucose homeostasis whereas the skin clock has a completely different task by regulating wound healing [33–35]. This is partially achieved by inducing the transcription of tissue-specific genes. For example Zhang et al. [36] comprehensively analyzed transcriptome data of 12 mouse organs collected across 2 days. They found that 43% of the quantified transcripts of protein-coding genes oscillate in at least one organ. This emphasizes the importance and extent of the circadian control in different organs across the body. Remarkably, there was little overlap between cycling transcripts in different organs (Fig. 3), whereas only transcripts of core clock genes cycled ubiquitously [36]. These findings highlight again that the circadian machinery drives tissue-specific outputs to control organ-specific physiology and metabolic needs across the day. Additionally, these findings raise the question: how is it possible that there is only a little overlap between cycling transcript in different organs, if the transcriptional mechanism is the same in all tissues?

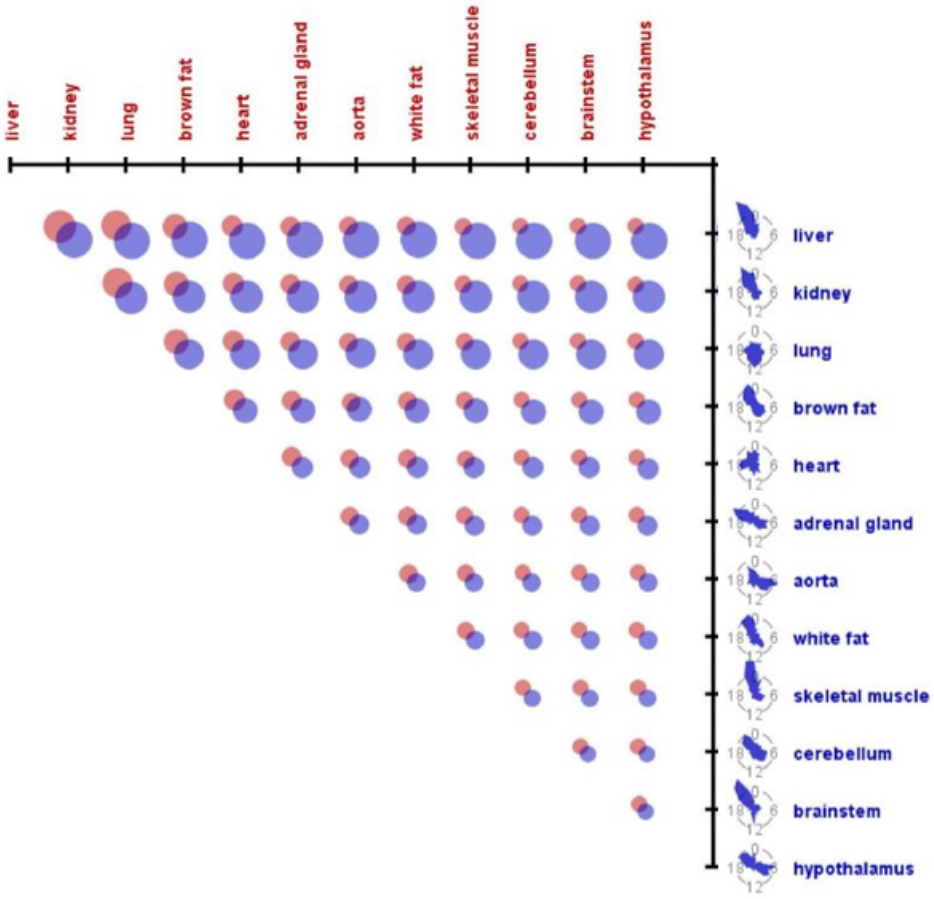

Fig. 3. Comparison of cycling transcripts across mouse tissues. Venn diagrams represent the oscillating transcripts identified in each indicated organ and the overlap between each pair (red and blue axis). Left panel shows circular histograms representing the distribution of peaks (phase) for all cycling transcripts in the indicated organ. Adapted from [36].

Interestingly, Trott and Menet performed a meta-analysis of different genome-wide sequencing datasets which suggests that the BMAL1:CLOCK mediated transcriptional control of core clock genes differ from other CCGs at the genome-wide level. They propose that BMAL1:CLOCK regulates transcription in each organ by interacting with tissue-specific and ubiquitously expressed transcription factors. They further imply that tissue-specific transcription factors enable the binding of BMAL1:CLOCK at tissue-specific enhancers, which leads to the recruitment of ubiquitously expressed transcriptions factors [27]. This supports the idea that CCGs expression relies on the interplay between the molecular clock and different signaling pathways in each organ to generate tissue-specific output signals. However, this interplay and how tissue-specificity is molecularly established remains unknown. One of the aims of this thesis was to develop a method to investigate this, in particular using mass spectrometry based proteomics to study the DNA-bound BMAL1:CLOCK complex in different tissues to thus identify tissue-specific clock components.

1.1.2.2 Sleep

Sleep is a fundamental process omnipresent in the animal kingdom. The time of sleep occurrence is controlled by the circadian clock, thus sleep can be also seen as a physiological and behavioral output of the circadian machinery [37]. However, the circadian clock alone is not sufficient to regulate sleep; accumulated evidences indicate that sleep is also modulated by sleep pressure or sleep homeostasis [38]. Thus, the circadian clock (Process C) and a homeostatic process (Process S) converge together to regulate sleep, which is the basis of the present model explaining sleep regulation, known as "the two- process model" (Fig. 4) [39,40].

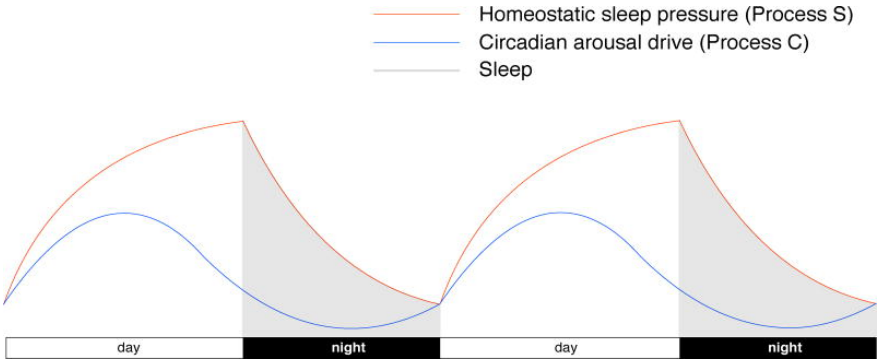

Fig. 4. Illustration of the regulation of sleep by the "two-process model" for a diurnal animal. Adapted from [41].

The course of both processes oscillates across a day (Fig. 4). The sleep pressure (S) accumulates during wakefulness and reaches its maximum right before sleep onset and its minimum at sleep offset, whereas process C indicates the propensity to fall asleep depending on the time of the day [39]. In contrast to the circadian rhythm, the homeostatic process is not self-sustainable when external stimuli are missing [37]. The oscillations of the homeostatic process arise from the sleep-wake distribution and sleep-wake is driven by neuronal groups in the brain. These neurons can act either by promoting sleep or by stimulating wakefulness. The sleep-promoting neurons are located in the hypothalamus as well as in the basal forebrain, whereas the neuronal groups associated with wakefulness have also been identified in the brainstem and the hypothalamus [42].

The physiological reasons that make sleep essential for all species are still under debate. One of the most famous hypotheses explaining the function of sleep has been proposed by Tononi and Cirelli. The so-called synaptic homeostasis hypothesis (SHY), postulates that functional "sleep is the price we have to pay for [synaptic] plasticity" and memory [43]. In other words, sleep restores synaptic strength, that is challenged during wakefulness. Hence, the course of process S (Fig. 4) can be read as synaptic potentiation during wake and synaptic downscaling during sleep [43].

To date, the typical method to track sleep is by using electroencephalogram (EEG) due to the neuronal activity involved and because sleep is molecularly rather unexplored. The EEG

measures the brain wave activity with specially placed electrodes. This allows to determine different stages of sleep based on the occurrence of waves and events characterized by their different frequency in the EEG (Fig. 5A) [44,45]. In this way sleep is distinguished into rapid eye movement (REM) and nonREM (NREM) sleep. NREM sleep is further discriminated into three stages: drowsiness (N1), light sleep (N2) and deep sleep (N3) (Fig. 5B). Stage N3 is also referred to as slow wave activity (SWA) stage [45,46]. The SWA in the EEG depicts membrane depolarization and hyperpolarization of neurons or in other terms oscillations in the membrane potential of cortical neurons [43].

A

Waves and events	Frequency range (Hz)
Delta	0.5–4
Theta	4–8
Alpha	8–13
Beta1	13–22
Beta2	22–35
Sleep spindles	12–14
K-complex	0.5–1.5

B

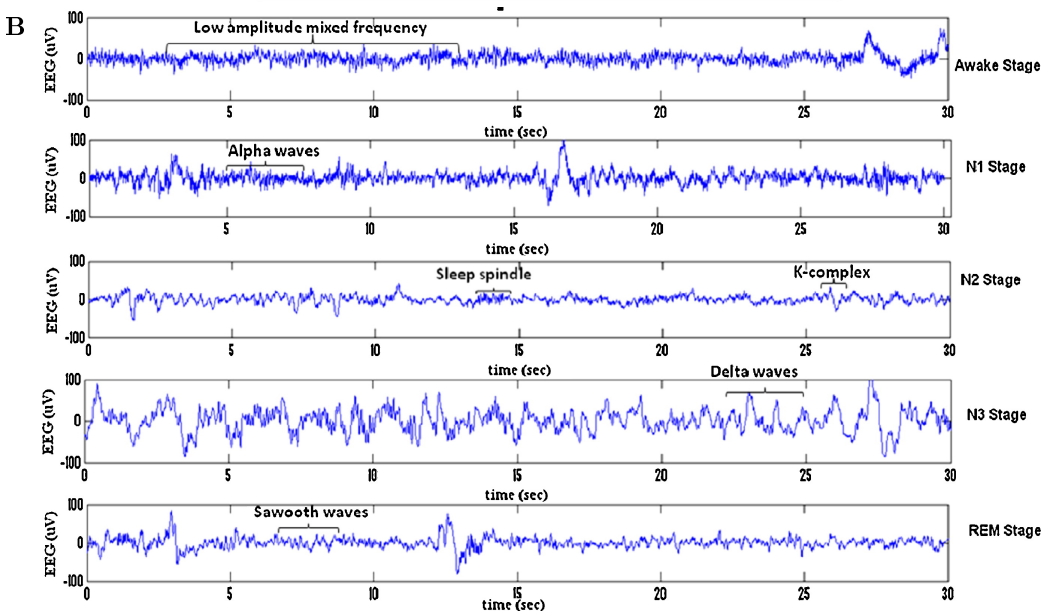

Fig. 5. Sleep stages. (A) Embedded waves and events in the EEG with their frequency range. **(B)** EEG signals with characteristic events for different sleep stages: the wake stage, N1, N2, N3 and REM stage. Adapted from [45].

In summary, sleep is a highly complex biological process of which fundamental pieces still need to be elucidated, in particular at the molecular level. Furthermore, little is known about how circadian and homeostatic signals interact molecularly to regulate synaptic activity and thus sleep.

Molecular Mechanism of Sleep

Despite a lot of studies, in flies, worms, fish and mammals, have been done to unravel the molecular mechanisms of sleep, no distinct sleep genes have been identified so far. It rather seems that sleep is regulated by multiple genes involved in other distinct important pathways such as neuronal functioning and the circadian clock, for which sleep is an output signal [47]. One example illustrating this is the role of the pineal gland produced hormone melatonin. Melatonin secretion is controlled by the circadian clock and its expression profile follows the course of process C (Fig. 4) which makes it a good marker for the internal time [48]. The melatonin onset is accompanied with the subjective feeling of sleepiness and decrease in body temperature [49, 50]. Melatonin, exogenously applied, phase shifts the circadian clock by increasing the sleep propensity and thus, acts as a putative Zeitgeber. That is why, melatonin is used to promote re-entrainment in jet-lag and shift work [51, 52].
Another example for the crosstalk between the circadian clock and sleep at the molecular level is the interaction of glutamate and adenosine having an effect on both, the sleep homoeostasis and the clock machinery. Light induces the release of glutamate at nerve terminals which boosts neuronal activity in the SCN. Notably, the injection of glutamate shows the same effect as light exposure, indicating that the light effect is mediated via glutamate action [53]. On the contrary, adenosine is known to be one of the major sleep-promoting molecules. The extracellular amount of adenosine increases during waking whereas the binding to the presynaptic A1 adenosine receptors leads to the inhibition of glutamate exocytosis which reduces neuronal activity [reviewed in [40]]. Sleep deprivation experiments in mice showed decreased photic phase shifts. This indicates that sleep pressure modulates the responsiveness of the circadian pacemaker to light [54]. Summarizing, sleep deprivation might induce blockage of the glutamate release and thereby reducing the strength of light on the circadian pacemaker [reviewed in [40]].
Other neurotransmitters have also been involved in sleep regulation either with wake-promoting actions (histamine, dopamine, acetylcholine and norepinephrine) or sleep-promoting function (gamma-aminobutyric acid (GABA) and serotonin) [reviewed in [37, 55]]. Many signal transduction pathways downstream of those neurotransmittors involve kinases such as protein kinase A (PKA) which is targeted by octopamine in Drosophila [56]. Furthermore, it has been shown, for example, that the ERK- MAPK pathway promotes sleep through, EGF activated ERK in Drosophila [57]. That is why kinases are a potential good molecular target to modulate sleep. Indeed, there are some studies showing sleep phenotypes in organisms with a deficient activity of certain kinases such as for the calmodulin-dependent kinases (CAMK2α and CAMK2β). Impairment of these kinases is reported to decrease sleep duration in mice [58].
Recent genetic screenings have also found ion channels as important sleep regulatory molecules,

which is not surprising, since ion channels are necessary for neuronal activity [47, 59]. Together and summarizing, a very complex picture is emerging indicating that sleep is regulated not by one distinct pathway but rather by converging and cross-talking signals from many different pathways. Still, the complete elucidation of the molecular events governing sleep will require many more experimental studies in the future.

1.2 Shotgun Proteomics

Proteins are the functional entities of cells and are therefore at the core of every biological process. The entirety of all expressed proteins at a given time and biological situation is referred to as "proteome". The experimental investigation of proteomes includes not only the determination of protein abundance but also the protein's state of modification, subcellular distribution and the interaction with other proteins or biomolecules [60].
Mass spectrometry (MS)-based methods such as shotgun or bottom-up approaches are commonly used to analyze proteomes in large-scale, reaching often the identification and quantification of thousands of proteins within the cell [60]. In shotgun proteomics, prior to analysis in the mass spectrometer, proteins are proteolytically digested, usually using trypsin or LysC (Fig. 6A) [61,62].
To reduce the complexity of the total mixture, peptides are separated by high performance liquid chromatography (HPLC), which separates the peptides based on their interaction strength with a stationary phase dependent on the gradually changing composition of a mobile phase (Fig. 6B) [reviewed in [63]]. For large-scale proteome analysis peptides are introduced to the mass spectrometer using electrospray ionization (ESI). This generates ions by volatilization of the solution that will be emitted into the mass spectrometer via the ion source [64,65]. In general a mass spectrometer consists out of three main components: an ion source, a mass analyzer and a detector [65].
In the mass spectrometer, first a so called MS1 or survey scan is done with all peptide-ions, eluting from the HPLC at a certain time point, by sending them to the mass detector (Fig. 6B). In case of the Q Exactive instrument, used for the thesis, this is an orbitrap [66]. In the orbitrap the ions oscillate around and along the long axis of a central electrode with a frequency that depends on their mass-to-charge ratio (m/z). The frequency is then used to obtain mass spectra and to calculate the peptide mass by applying Fourier transformation [67,68]. Thus, the MS1 scan provides information of the accurate masses of all eluting peptides at a certain time point. Next, a number (usually 10-15) of MS/MS (MS2) scans are performed to isolate and then to fragment the most abundant peptides from the MS1 scan (Fig. 6B and C). There are different fragmentation methods, a common on is higher-energy collisional dissociation which is used for the MS methods in this thesis [reviewed in [60,68]]. During this process the peptide back bone is cleaved resulting in a series of N-terminal and C-terminal fragments, termed b-ions and y-ions, that are then send to orbitrap for detection [69]. The obtained fragmentation spectra allow to determine the distinct sequence of a peptide. The alternation between MS and MS/MS spectra is called tandem mass spectrometry [reviewed in [60,68]]. The tandem mass spectrometry approach, described above, is thus applied to obtain the peptide intensity (using MS1 scans) and peptide sequence (using the MS/MS spectra). The raw files, created during MS-analysis, need to be then processes to

identify and quantify the measured peptides/ proteomes. For this reason different analytical tools have been developed. One of these software tools is MaxQuant which is an in-house developed freely available software to analysis large MS-data sets (Fig. 6 C).
The implemented algorithms and search engines compare the measured mass with theoretical masses from a database of the peptide fragments. The thereby identified peptide sequences are then matched to an organism-specific protein sequence database to compare the peptide sequence to the corresponding protein. In this way post-translational modifications can also be detected and identified, based on the specific mass shift that the particular modification causes on the corresponding peptide sequence [70, 71].

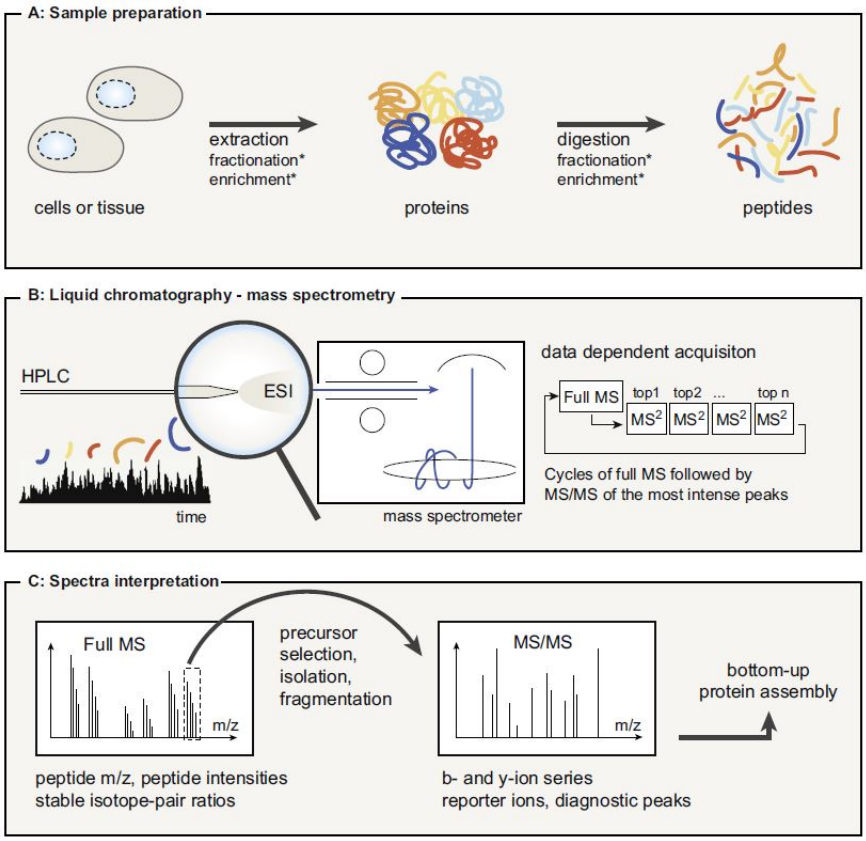

Fig. 6. Standard proteomic shotgun workflow. **(A)** Schematic representation of a standard proteome sample preparation for MS analysis using specific endonucleases to digest isolated proteins in peptides. Depending on the application, fractionation and/or enrichment steps can be additionally applied at the protein and peptide level. **(B)** The complexity of the peptide mixture is reduced by high performance liquid chromatography (HPLC). The eluting peptides are then electrosprayed directly into the mass spectrometer by electrospray ionization (ESI). Following this, peptides are measured in data dependent acquisition mode by first acquiring a full MS scan followed by the fragmentation of the 10-15 most abundant peptide ions (MS/MS spectra) for each MS scan. **(C)** Full MS scans provide information about peptide mass and intensity. Further fragmentation of selected peptides leads to MS/MS spectra which together with the peptide masses are used to find the peptide sequence which is matched to corresponding proteins by searching against an organism specific protein sequence database. Adapted from [60].

MS is the state-of-the-art technique to identify proteins in a complex mixture. However, not only the identification but most often the quantification of proteins is as well of biological interest. Protein quantification can be achieved using label-based and label-free methods. Label-based quantification itself can be divided in two groups: chemical or metabolical labeling. Chemical labeling such as TMT (Tandem Mass Tag) [72] and iTRAQ (isobaric tags for relative and absolute quantitation) [73] is introduced after the tryptic digestion of the proteins at the peptide level whereas metabolic labeling like SILAC (stable isotope labeling by amino acids in cell culture) [74] is done at the protein level. The labeling causes a specific mass shift in the proteins and/or peptides which can be distinguished from non-labeled or differently labeled peptides in the mass spectrometer. The ratio of the differently labeled peptide intensities gives information about the relative amount of the proteins of interest compared to the control. The labeled peptides in both conditions are comparable, because they behave the same in respect to chromatographic separation as well as ionization due to their chemical equivalence [reviewed in [60]].
Label-free quantification (LFQ) relies on a computational approach without any experimental label; therefore it can be applied to any cells and tissue including human samples. Moreover, the lack of the labeling step makes the sample preparation workflow simpler, cheaper and enables high-throughput measurements due to the unlimited sample numbers. The improvement of the LFQ algorithms made this approach a widely accepted method for quantitative MS [75]. In label-free shotgun proteomics the quantification can be based on identification results and ion intensities. Various methods have been developed using these two data types to infer relative and absolute protein quantification [reviewed in [76]]. Relative quantification by identification-based methods includes mainly counting-based approaches such as peptide or spectral counting. By now, intensity based LFQ methods have been shown to be more accurate and precise [reviewed in [76]]. They are based on the fact that the intensities of individual peptide peaks linearly correlate with the peptide concentration [77]. A sophisticated intensity-based LFQ algorithm is for example implemented in the MaxQuant software, which also corrects for variations introduced by sample preparation and instrument performance using peptide retention time alignment and intensity normalization with background proteins present in all samples [75, 78].

1.2.1 Interaction Proteomics

Proteins carry out a huge amount of functions within cells by interacting with different biomolecules such as other proteins, nucleic acids, lipids, carbohydrates and metabolites. One protein can be in different complexes at different times and thereby building a huge flexible interacting network of macromolecular complexes to ultimately regulate biological functions [79]. The sum of all molecular interactions of a biological system is referred to

as "interactome" [60]. The dissection of the cellular interactomes is essential for the understanding of how cells maintain their functions and respond to different stimuli.
In the past, mainly genetic readouts such as the phage display and the yeast two hybrid assay were used to study binary interactions [80, 81]. However, these methods are purely based on direct interactions in a non-cellular context and thus miss essential information about time, space and the actual composition of the whole protein complex within cells, which is critical for biological function. MS-based interaction proteomics is a powerful technique that enables to overcome these limitations. Consequently, whole protein complexes can be enriched or purified under physiological conditions allowing the unbiased identification of all complex members in space and time. The enrichment is usually done by using an antibody directly against the "bait" protein itself or against an epitope tag (Fig. 7). Antibodies against endogenous proteins are of limited availability and might also show cross-reactivity, thus epitope tags such as FLAG and the green fluorescent protein (GFP) tags are widely used with the limitation that protein interactions and/or protein localization might be affected by the introduction of the tag sequence [82–84].

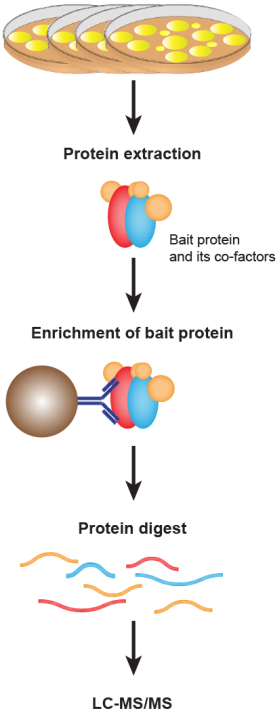

Fig. 7. Single-step AP-MS workflow. First, proteins are isolated from cells or tissues. Second, antibodies coupled to an affinity matrix e.g. magnetic beads target endogenous proteins or epitope tags. Finally, after washing steps (not illustrated) enriched bait protein containing complexes are digested and analyzed using high resolution LC-MS/MS.

Antibodies against the bait can be coupled to an affinity matrix such as magnetic or agarose

beads allowing the purification of the bait protein and its interacting partners (Fig. 7). After washing steps to remove unspecific binders, the specific interactors are eluted and analyzed by LC-MS/MS. This workflow is commonly termed affinity purification mass spectrometry (AP-MS) [82,85,86].

The identification of true specific interactions has remained a challenge for a long time due to non-specific binding of proteins to the affinity matrix or antibody and the high sensitivity of mass spectrometers allowing the identification of background binders [87,88]. The introduction of quantitative MS and the development of suitable background normalization algorithms in the last years allow now the easier dissection of true interactors and background binders with fast and low stringent single-step purifications (Fig. 7).

Specific interactions can be simply identified by comparing affinity purification of the bait against a control [89,90]. Controls are for instance immunoprecipitations done with an irrelevant antibody like immunoglobuline G (IgG) or immunoprecipitions with the specific antibodies using samples lacking the bait protein or epitope tag. Changes and dynamics in protein complex composition, important for many biological questions, can be addressed by doing and comparing immunoprecipitations under different conditions using label-free and label-based approaches [89–93].

Although quantitative proteomics enables to study low abundant complexes, very dynamic complexes are still difficult to identify and characterize. This issue is mainly solved by using cross-linking MS workflows. In these approaches chemical cross-linkers are used to stabilize or "freeze" complexes in their current composition. One of the most commonly used cross-linker is formaldehyde, which is able to permeate intact cell walls and membranes therefore enabling the stabilizing of complexes in their native environment [94]. Formaldehyde preferably crosslinks lysine residues and, to a lesser extent, tryptophan, cysteine as well as peptides N-termini [95,96]. Since lysines are known to mediate the interaction of proteins with DNA, formaldehyde is also widely applied to study protein-DNA interactions [97,98]. The combination of cross-linking followed by chromatin shearing and subsequent AP-MS is the basis of chromatin immunoprecipation MS (ChIP-MS); a method that allows to study complexes of proteins such as transcription factors, often low abundant, bound to the chromatin (Fig. 8) [99].

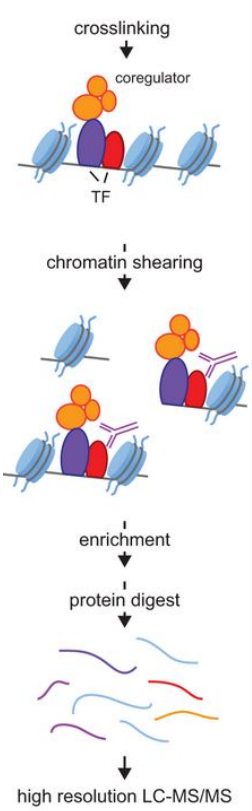

Fig. 8. Schematic illustration of the ChIP-MS workflow. First, the complexes residing on the chromatin are crosslinked using formaldehyde. Second, the chromatin is sheared by sonication. Third, the complexes of interested are enriched using antibodies against endogenous transcription factors (TF) or against epitope tags. Finally, enriched proteins are digested and analyzed using high resolution LC-MS/MS. Adapted from [99].

1.3 Large scale Proteomics Studies in the Circadian Field

Most of the large-scale circadian studies focus on the characterization of rhythmic gene expression using microarrays and, more recently, RNAseq. However, due to technical limitations the exploration of circadian dynamics of the proteome has been more limited [100]. The first MS-based proteomic study in the circadian field used 2-dimensional-difference gel electrophoresis (2D-DIGE) to separate proteins by their isoelectric point and mass, followed by MS-analysis. In this way the first mammalian "circadian proteome" of mouse liver reported the finding of 642 protein gel spots of which 60 showed circadian oscillations. These 60 rhythmic spots were further analyzed by MS resulting in a final list of 49 rhythmic proteins [101].

Improvements in MS technology allowed now deeper and more comprehensive large-scale studies, reaching the identification and quantification of thousands of proteins in different tissues. Depending on the tissue, circadian cycles of the total proteome reached 2-6%, for example 2.2% in mouse SCN [102] and 5-6% in mouse liver [103,104]. The comparison of the rhythmic transcriptome and proteome of the SCN revealed that a large number of rhythmic proteins lack temporal regulation at the transcriptional level [102]. In contrast, in liver there was a larger overlap between oscillating transcripts and their proteins, however the protein abundance peak did not correlate to its mRNA level and overall the time window between transcript and protein peak extended several hours [103, 104]. These findings highlight the importance to study the proteome, since proteins are the functional entities of the cell and their abundance cannot be determined by their transcript levels.

Moreover, the function of proteins also depends on their cellular location which can dynamically change across the day. Indeed studies investigating proteome rhythms in mitochondria and nuclei, isolated from mouse liver, reported a higher number of cycling proteins, 29% in mitochondria [105] and 10% in nuclei [106], compared to the 6% found in total liver [103,104]. Thus temporal control of protein function could be achieved by regulating its cellular localization rather or alternatively to control its abundance. Therefore, proteomic experiments around the clock performed in cellular compartments could provide important information about how metabolic processes are temporally controlled.

Protein abundance is often used as a proxy of protein function, however protein activity can be modulated by PTMs in addition, or in the absence, of protein abundance changes. Thus, recently, several groups have examined dynamics of different PTMs in a large-scale manner to investigate how circadian outputs downstream of protein expression temporally regulate metabolism and physiology [107–110]. Phosphorylation is the most studied PTM so far because it is relatively easy to enrich for phosphopeptides [111]. The enrichment method in combination with advanced MS has enabled to identify global, time-dependent phosphorylation changes in different murine tissues at different degrees. For example in liver 25% [107]

of the quantified phospeptides showed oscillation, whereas in the hippocampus only 5% [108] were reported to show rhythmicity. Interestingly, in both organs there was either no overlap between the rhythmic phosphoproteome and proteome or a great divergence between the phases of cycling proteins and phosphopeptides. This suggests that phosphorylation plays an important role, downstream of protein abundance, in fine tuning circadian controlled signalling pathways in both organs [107, 108]. For instance, cycling phosphoproteins in the hippocampus were largely involved in synaptic function and cytoskeletal organization, indicating that synapses are a cellular compartment where the circadian clock plays an important molecular role [108].

Since, no specific proteomic studies have been performed so far in isolated synapses in the brain, one aim of this thesis was to study the circadian proteome and phosphoproteome of forebrain synapses around the clock.

2 Results

2.1 Chromatin Immunoprecipitation followed by Mass Spectrometry allows Characterization of the Core Clock Complex in different Tissues

2.1.1 Project Aim and Summary

The discovery of the basic molecular machinery controlling the circadian rhythm was awarded with the Nobel prize in 2017. The basic molecular machinery consists of transcriptional-translational feedback loops with the core clock transcription factors BMAL1 and CLOCK driving the transcription of many genes including their own repressors CRYs and PERs. Several studies have uncovered additional proteins interacting with different core clock components and thus regulating circadian gene expression. However, until now only fragments of this highly complex interaction network are known. For example, it is still not fully understood how exactly the composition of the core clock complex changes over time and how potentially the "same" molecular machinery regulates rhythmic gene expression in different tissues.

MS-based quantitative proteomics offers methods to study protein interactions that dynamically change under different physiological conditions. I applied interaction proteomics to study, in a time- and tissue-dependent manner, the protein composition of complexes containing the core clock transcription factors CLOCK and BMAL1. In particular, one aim of my thesis was to optimize a chromatin immunoprecipitation (ChIP) method followed by MS to study the BMAL1:CLOCK complex at the chromatin.

One of the critical steps in this project was to find antibodies recognizing endogenous CLOCK and BMAL1 that are suitable for ChIP-MS. I chose to test different commercially available antibodies in the U-2 OS cell line, a widely used cell culture model in the circadian field. After the adjustment of some experimental parameters, I tested and further optimized the method for mouse tissues as well.

In both, U-2 OS cells and mouse tissues, I was able to efficiently precipitate, along with the bait proteins CLOCK and BMAL1, other known core clock proteins such as CRYs. During the testing and optimization of this method, I already observed that pulled down proteins varied according to the bait protein, the tissue and the time point, supporting the suitability of this method for the potential success of this project.

In conclusion, the established ChIP-MS method and the tested antibodies indicated that this is an effective application to study the BMAL1 and CLOCK complex composition in a time- and tissue-dependent manner.

2.1.2 Contribution and Publication

For this project I established and performed the ChIP-MS protocol in cell lines as well as in diverse mouse tissues and thereby tested commercially available antibodies targeting endogenous CLOCK and BMAL1.
Michael Wierer, a postdoc in Matthias Mann's Lab, developed the ChIP-MS protocols and helped adapting the method to study the core clock complex. I performed the preparation of all ChIPs, the MS data acquisition and the analysis under the supervision of Maria Robles. During the optimization of the method in U-2 OS cells, one of the significant hits in the CLOCK-ChIPs caught the interest of Achim Kramer (Charité Berlin), who is a collaboration partner and member of my thesis advisory committee. At this time, his lab had identified Transportin 1 (TNPO1) as potential novel regulator for the nuclear localization of PER1. We provided interaction proteomics data to complement their study to finally show that TNPO1 interacts with clock proteins and thus modulates the molecular mechanism of the clock. This work resulted in a publication in PLOS Genetics (see Appendix):

The non-classical nuclear import carrier Transportin 1 modulates circadian rhythms through its effect on PER1 nuclear localization

Sandra Korge, Bert Maier, Franziska Brüning, Lea Ehrhardt, Thomas Korte, Matthias Mann, Andreas Herrmann, Maria S. Robles, Achim Kramer

PLoS Genet. 2018 January; 14(1)

The unpublished main findings of the ChIP-MS method adjustment to study the interaction complexes of CLOCK and BMAL1 in time and space are described in detail in the following preliminary manuscript.

2.1.3 Preliminary Manuscript

2.1.3.1 Experimental Procedure

Animals and cells

Male C57BL/6 mice were sacrificed either 8-9 hours after the lights were turned on (ZT8-9) or at ZT6. Tissues were harvested and snap-frozen.
U-2 OS cells (gift from Achim Kramer, Charité Berlin) where grown in DMEM containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were harvested using trypsin and were washed once with PBS.

Chromatin immunoprecipitation followed by mass spectrometry

ChIP-MS was performed with mouse liver and kidney as well as unsynchronized U-2 OS cells as described [112, 113] with modifications.

Snap-frozen murine tissues were cut into pieces of about 150mg of weight and minced in cross-linking solution (PBS containing 1% formaldehyde (#F8775, Sigma)) using a glass dounce homogenizer with tight pestle for 10min. Cross-linking reaction was quenched by adding glycine to a final concentration of 125mM for 5min at room temperature (RT). Following this, cross-linked cells were pelleted by centrifugation (500g, 5min, 4°C) and washed two times with cold PBS by repeating the re-suspension of the pellet in PBS followed by centrifugation (500g, 5min, 4°C) and removal of the supernatant. After this minced and cross-linked tissue was snap-frozen. Next, tissue pellets were incubated in 1ml cold Fast IP buffer (150mM NaCl, 5mM EDTA pH 7.5, 5mM Tris pH 7.5, 1% Triton X-100, 0.5% NP40) for 10min on ice. During that time, the suspension was passed through a syringe (24G) and after incubation cleared by centrifugation (12,000 rpm, 4°C). The supernatant was aspired and the whole process of adding 1ml cold Fast IP buffer to the pellet, 10 min incubation on ice, passing suspension through the syringe and centrifugation was repeated.
After removing the supernatant for the second time, 1ml cold Shearing buffer (1% SDS, 10mM EDTA pH 8, 5mM Tris pH8) supplemented with protease inhibitors (#4693159001, Roche) was added to the nuclei pellet. Samples were then transferred to falcons (#352095). Chromatin was sheared to an average size of 300bp using a Bioruptor (Diagenode) on high power settings. Sonicated chromatin was cleared by centrifugation (12,000rpm, 4°C, 10min) and then diluted 1:10 with cold Dilution buffer (0.01% SDS, 1% Triton, 1.2mM EDTA pH 8, 16.7mM Tris pH 8, 167mM NaCl) supplemented with protease inhibitors (#4693159001, Roche).
5 μg of antibody was added to 1mg of protein per sample and incubated overnight at 4°C on a rotation wheel. For each antibody the experiment was performed in at least triplicates. After immunoprecipitation with antibodies against CLOCK (#5157, Cell Signaling; #ab3517, Abcam), BMAL1 (#14020, Cell Signaling) and IgG (#2729, Cell Signaling) as control, antibody-bound complexes were captured to A- and G-coupled magnetic beads (Magna ChIP, #16-663, Sigma-Aldrich) that were then washed three times with cold wash buffer A (50mM HEPES pH 7.5, 140mM NaCl, 1% Triton), one time with cold wash buffer B (50mM HEPES pH 7.5, 500mM NaCl, 1% Triton) and two final washes with cold TBS.
ChIP-MS in unsynchronized U-2 OS was performed as described above except that the cell pellets were incubated in cross-linking solution directly after the cells were harvested. Moreover, snap-frozen, cross-linked pellets were directly lyzed in cold Cell-IP-Buffer (50mM Tris-Cl pH 8.0, 100mM NaCl, 5 mM EDTA pH 8.0, 1.7% Triton X-100, 1% SDS), sonicated, 5μg of antibody was added to 1mg of protein per sample without further dilution. Samples were then processed as described above.
Following the washing steps, proteins were on-bead digested as described [83]. Briefly, beads were first incubated with 50μl elution buffer 1 (2M Urea, 50mM Tris-HCl pH 7.5, 2mM DTT, 20g/ml trypsin) at 37°C for 30min with rapid agitation (1300rpm). The eluates were sepa-

rately saved and later combined with the supernatant from the second elution which was done by adding 50μl elution buffer 2 (2M Urea, 50mM Tris-HCl pH 7.5, 10mM Chloroacetamide) to the beads for 5min at the same temperature. The digestion of the combined eluates was continued over night at RT. Peptides were acidified with TFA to a final concentration of 1% and desalted using StageTips with two layers of styrene-divinylbenzene–reversed phase sulfonated (SDB-RPS; 3M Empore) as reported [114], washed twice with wash buffer (0.2% TFA) and once with isopropanol containing 1% TFA. Peptides were eluted from the SDB-RPS material by adding 60μl of SDB-RPS elution buffer (80% ACN, 1.25% NH4OH [25%, HPLC grade]) and immediately concentrated in a SpeedVac for 30min at 45°C. Finally, concentrated peptides were resuspended in a buffer containing 2% ACN and 0.1% TFA prior to LC-MS/MS analysis.

LC-MS/MS analysis and data processing

Half of the peptide mixture (approximately 400ng) was loaded onto a 50cm reversed-phase column (diameter 75μM; packed in-house with 1.9μM C18 ReproSil particles [Dr. Maisch GmbH]). The column oven temperature was maintained at 60°C and the column was mounted to an EASY-nLC 1000 system (Thermo Fisher Scientific). The peptides were eluted with a binary buffer system consisting of buffer A (0.1% formic acid) and buffer B (80% ACN and 0.1% formic acid). A gradient was run from 5% to 60% buffer B over 120min with a flow rate of 250nl/min. Peptides were analyzed in a Q Exactive HF-X (mouse liver ZT8-9) and Q Exactive HF (unsynchronized U-2 OS cells and mouse tissues ZT6; parameters described in [115]) mass spectrometer (Thermo Fisher Scientific) coupled to the nLC. Briefly for Q Exactive HF-X runs, full scans (300-1,650 m/z, R = 60,000 at 200 m/z) were obtained at a target of 3e6 ions. The 15 most abundant ions were selected and fragmented with higher-energy collisional dissociation (target 1e5 ions, maximum injection time 60ms, isolation window 1.4m/z, normalized collision energy 27%) followed by the detection in the Orbitrap (R = 15,000 at 200m/z). Raw MS data files were processed using the LFQ-algorithm of MaxQuant (internal versions 1.6.0.8 and 1.6.0.8) with the Andromeda search engine using false discovery rate (FDR)$<$ 0.01 at protein and peptide level [78]. Variable modifications for oxidized methionine (M) and acetylation (protein N-term) as well as a fixed modification for carbamidomethyl (C) were included in the search. The standard "match between runs" option was enabled with a matching time window of 0.7min. The UniProt database from mouse and human (September 2014) was used for protein and peptide identification. Each raw file was treated as one experiment and only replicates of the same antibody and condition were analyzed by matching between each other.

Statistical analyses

The Perseus software (version 1.6.10.50 for tissues and 1.5.4.1 for U-2 OS) was used for statistical analyses [116]. Briefly, the analytical steps were the following: first, potential contaminants as well as reverse sequences were removed from the data set. Second, label-free intensities were transformed to the logarithm with base 2. Third, entries that contained less than n-1 numerical values (n=number of replicates for one group) in at least one group were filtered out. In this case a group combines the replicates performed with each used antibody (IgG (Cell Signaling (CS)), CLOCK (Abcam), CLOCK (CS) and BMAL1 (CS)). Missing values were imputed, using a random value from a window of the normal intensity distribution of each sample defined with a down shift of 1.8 and a width of 0.3 (as described in [117]).
To find interesting clusters of antibody-specific identified interactors, permutation-controlled analysis of variance (ANOVA, FDR<0.05, s0$=0$, randomizations=250) was used. The LFQ intensities of the ANOVA-significant hits were z-scored and clustered according to their Euclidean distance while the grouping of the column tree (ChIP-samples) was preserved.
For the identification of significant outliers in individual ChIPs a permutation-controlled Student's t-test was performed. For this, replicates of each ChIP-condition using specific antibodies were separately filtered for valid values with the control IgG-ChIPs, only allowing entries with certain quantified numeric values (at least n-1 values in a group). FDR and s0-value were individually adjusted for each test. Figures and plots were generated using the R software (version 3.6.3) environment and RStudio (version 1.2.5033) with its packages ggplot2 (version 3.3.0), ggrepel (version 0.8.2), dplyr (version 0.8.5) as well as stringr (version 1.4.0).

2.1.3.2 Results and Discussion

ChIP-MS in unsynchronized U-2 OS as promising tool to study core clock interactions

I started to establish the ChIP-MS protocol in unsynchronized U-2 OS cells by testing commercially available antibodies recognizing endogenous BMAL1 and CLOCK as well as a non-specific IgG-antibody as control. After performing ChIP-MS, the LFQ intensities obtained for all immunoprecipitated proteins in all conditions were compared using ANOVA testing. Hierarchical clustering of the LFQ intensities from the ANOVA significant hits resulted, as expected, in antibody- and bait-specific clusters. I focused on the cluster with hits with high, significant intensities in the pulldowns with BMAL1 and CLOCK antibodies in comparison to the IgG control pulldowns. In doing so, I tried to reduce false positives due

to unspecific binding to one of the antibodies and to thus increase the chances to discover known core clock components which bind to BMAL1:CLOCK to validate the method (Fig. 9).

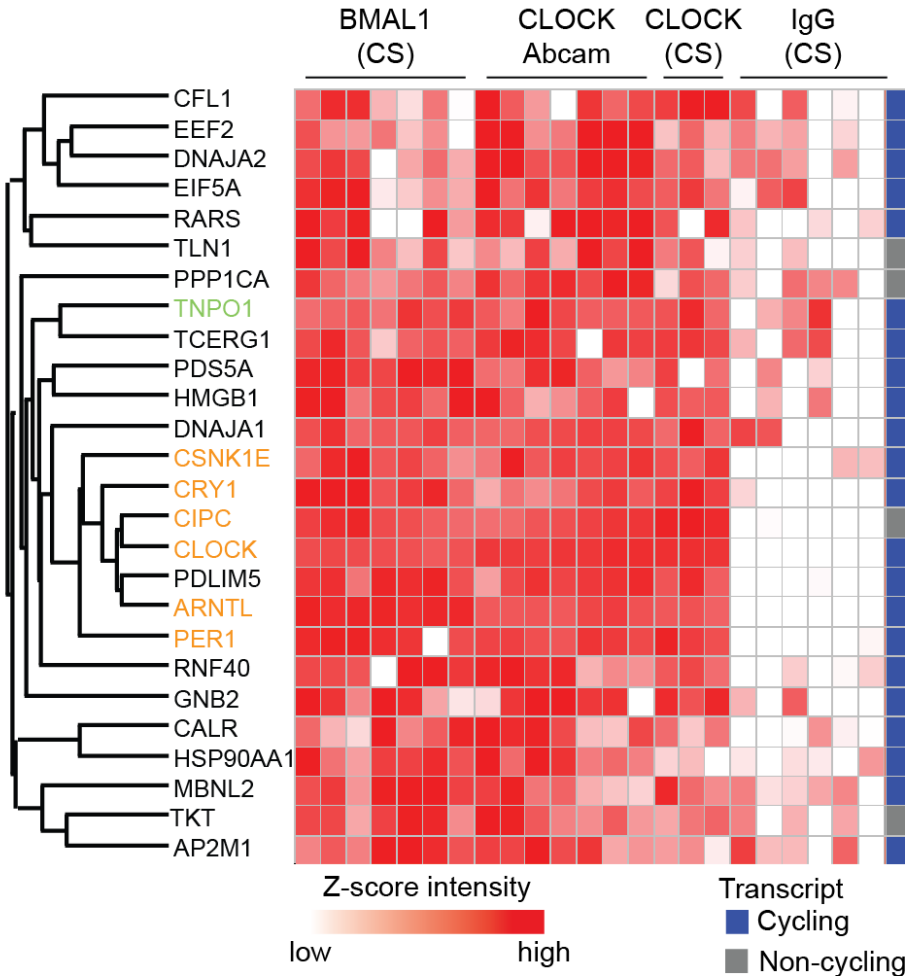

Fig. 9. ChIP-MS in unsynchronized U-2 OS cells. Heat map showing the intensities (log2, z-scored) of proteins (rows) which are statistically significant (ANOVA, FDR<0.05) between the ChIPs (columns) using specific antibodies (against BMAL1 and CLOCK) and the IgG control. Proteins known to interact with BMAL1:CLOCK are colored in orange. Last column indicates if this protein has a rhythmic transcript in mouse liver based on CircaDB [118].

This cluster contained a number of already known BMAL1:CLOCK interactors: PER1 [119], CRY1 [17], CSNK1E [120] and CIPC [121] (Fig. 9, highlighted in orange), indicating that the antibodies as well as the protocol are suitable to perform ChIP-MS in U-2 OS cells. Moreover, the data also showed that the method allows to identify direct and indirect interactors. This was the case of the clock-interacting pacemaker (CIPC). As the name indicates, CIPC binds directly to CLOCK but not to BMAL1 [121], our data sustained this finding since CIPC was more abundant in the CLOCK-ChIPs compared to BMAL1-ChIPs; hence CIPC was presumably present in the BMAL1 pulldowns due to its interaction with CLOCK and could be designated as a "secondary interactor". The same might be true for CSNK1E, a critical

kinase of the molecular mechanism of the clock machinery. It is known that CSNK1E interacts with PER1, and since PER1 was present in all ChIPs except in the control, it is possible that CSNK1E binds to BMAL1:CLOCK via PER1, although I cannot exclude CSNK1E as a direct interactor of BMAL1:CLOCK [120]. However, it has not been reported so far that CSNK1E forms a complex with BMAL1:CLOCK. The nature of this interaction could be validated in further experiments as it was done for another significant hit, TNPO1 (Fig. 9, green). In collaboration with Achim Kramer's Lab at the Charité in Berlin, we showed that TNPO1 influences the circadian period by affecting the translocation of PER1 in the nucleus [115], therefore TNPO1 will be another indirect interactor of BMAL1:CLOCK via PER1. Similarly, protein phosphatase PPP1CA, another significant hit, could be a secondary interactor as well, because it is known to dephosphorylate PER1 [122].
The remaining protein hits are to date unknown as interactors of BMAL1:CLOCK, however it is very promising that the majority of them (as indicated by the blue bar in Fig. 9) are rhythmically controlled at the transcriptional level *in vivo* (in mouse liver). Further functional assays would allow to validate those interactors and to identify their role in the molecular mechanism of the clock.
In conclusion and in summary the following findings denote ChIP-MS as a very promising method for the aims of the project: (1) the bait endogenous proteins and their partners were the most highly significant pulled down hits, (2) known validated interactors were also present among the significant hits and (3) presumably secondary interactors could also be significantly detected.

ChIP-MS in mouse tissue as a promising tool to study tissue-specific and temporal core clock interactions

I next focused my work on optimizing the ChIP-MS method *in vivo* using the already tested commercial antibodies recognizing endogenous CLOCK and BMAL1. For that I used mouse liver collected at Zeitgeber time (ZT) 8-9 corresponding to the transcriptional activation phase when BMAL1 and CLOCK are reported to be maximally bound to DNA [123]. The ChIP-MS results indicated that the antibodies and the method are suitable to enrich BMAL1:CLOCK complexes also *in vivo*, in particular in liver. The bait proteins, CLOCK and BMAL1, were the most significant outliers when compared to the controls done with IgG (Fig. 10A and B). Furthermore, other known core clock components such as CRY1, CRY2, CSNK1D/E and CIPC were also immunoprecipitated. Interestingly, CRY1 was significantly enriched in both CLOCK- and BMAL1-ChIPs whereas other known clock proteins predominantly bound to one of the transcription factors. The difference between the ChIPs can be visualized by plotting the t-test fold change intensity value obtained when individually comparing the CLOCK- and BMAL1-ChIPs to the IgG control (Fig. 10C). Thus, it can be easily observed that, for example, CRY2 was significantly pulled down with

BMAL1 but not with CLOCK, indicating that it may interact exclusively with BMAL1 (Fig. 10C). The opposite is seen for CIPC which is only significantly enriched when pulling down CLOCK (Fig. 10A and B) as originally described [121] and, as mentioned above, also observed in U-2 OS ChIPs. Interestingly, although the used tissue corresponded to the activation phase when BMAL1 and CLOCK are maximally bound to DNA [123], both repressors, CRY1 and CRY2, were detected to interact with the bait proteins. This is in line with the ChIPseq study of Koike et al. in which they found CRY1 and CRY2 bind to BMAL1:CLOCK occupied promoters in the early repression phase which overlaps with the late activation phase. In contrast, PERs are only associated to BMAL1:CLOCK in the late repression phase [123], thus PERs should not be present in the BMAL1 and CLOCK complexes at ZT8-9, as the ChIP-MS preliminary data indicated.

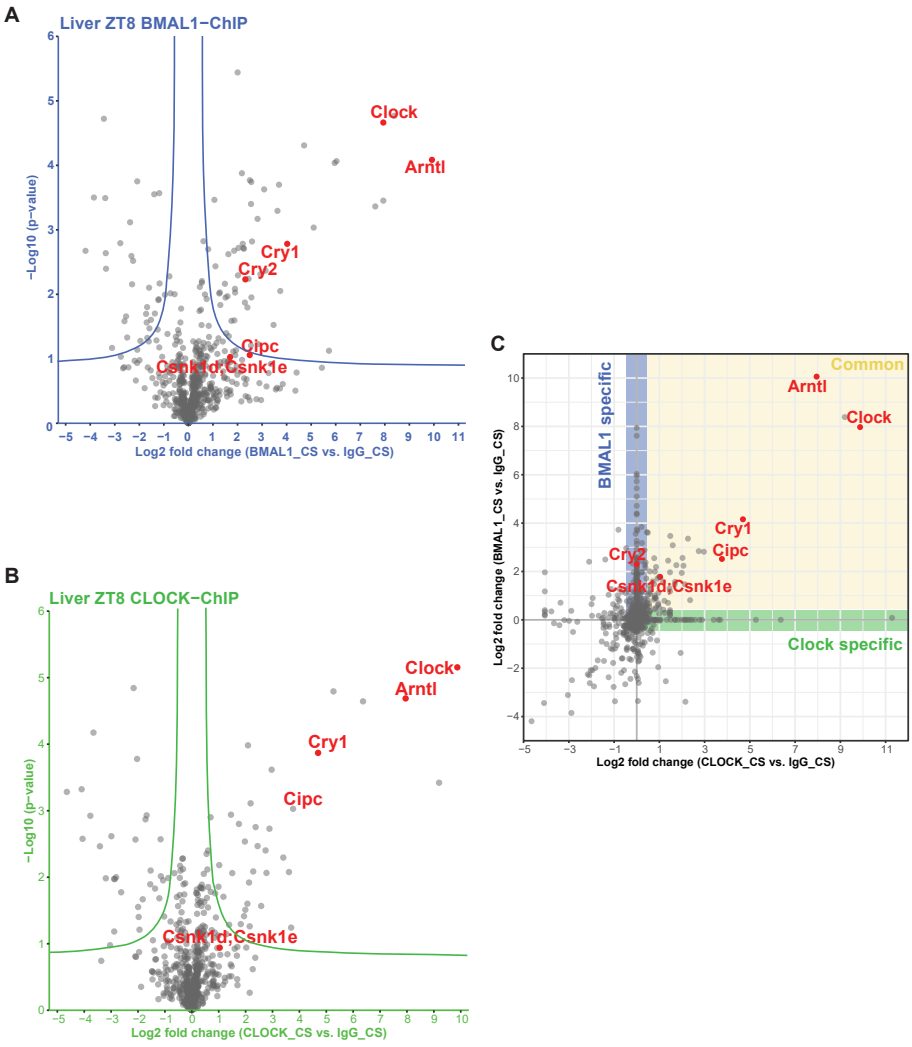

Fig. 10. ChIP-MS in mouse liver at ZT8-9. **(A)** Volcano plot representing the Log2 fold change and -Log10 p-value between the ChIPs using the BMAL1-antibody and the IgG-control (FDR<0.06, s0= 0.3). **(B)** Volcano plot as in (A) but the result of the ChIPs using the antibody against CLOCK is ploted against the IgG-control (FDR<0.1, s0=0.3). **(C)** Log2 fold changes from (A) and (B) are ploted against each other. Known clock components are labeled in red.

Next, I was interested whether I could detect time-dependent variation in the BMAL1:CLOCK protein-complex composition by using liver tissue harvested at a different time point, 2 hours early at ZT6. I performed ChIP-MS as before, using the same antibodies against BMAL1 and CLOCK as well as the IgG-control. I could successfully detect BMAL1 and CLOCK as well as other clock proteins similar to 2-3 hours later at ZT8-9 (Fig. 11). Surprisingly, NPAS2, a CLOCK paralogue that is reported to bind BMAL1 in absence of CLOCK as a molecular compensation mechanism, was significantly pulled down with BMAL1 at this new time point [124, 125]. Further experiments will need to be performed to find out whether there is an additional transcriptional complex in liver at ZT6 containing NPAS2 binding to BMAL1 even in the presence of CLOCK. Moreover, CSNK1D/E, found in the previous ChIPs at ZT8-9, could not be detected in either of the pulldowns at ZT6 (Fig. 11) suggesting that the interaction of CSNK1D/E with BMAL1:CLOCK takes place in the later activation phase at ZT8-9 in mouse liver.

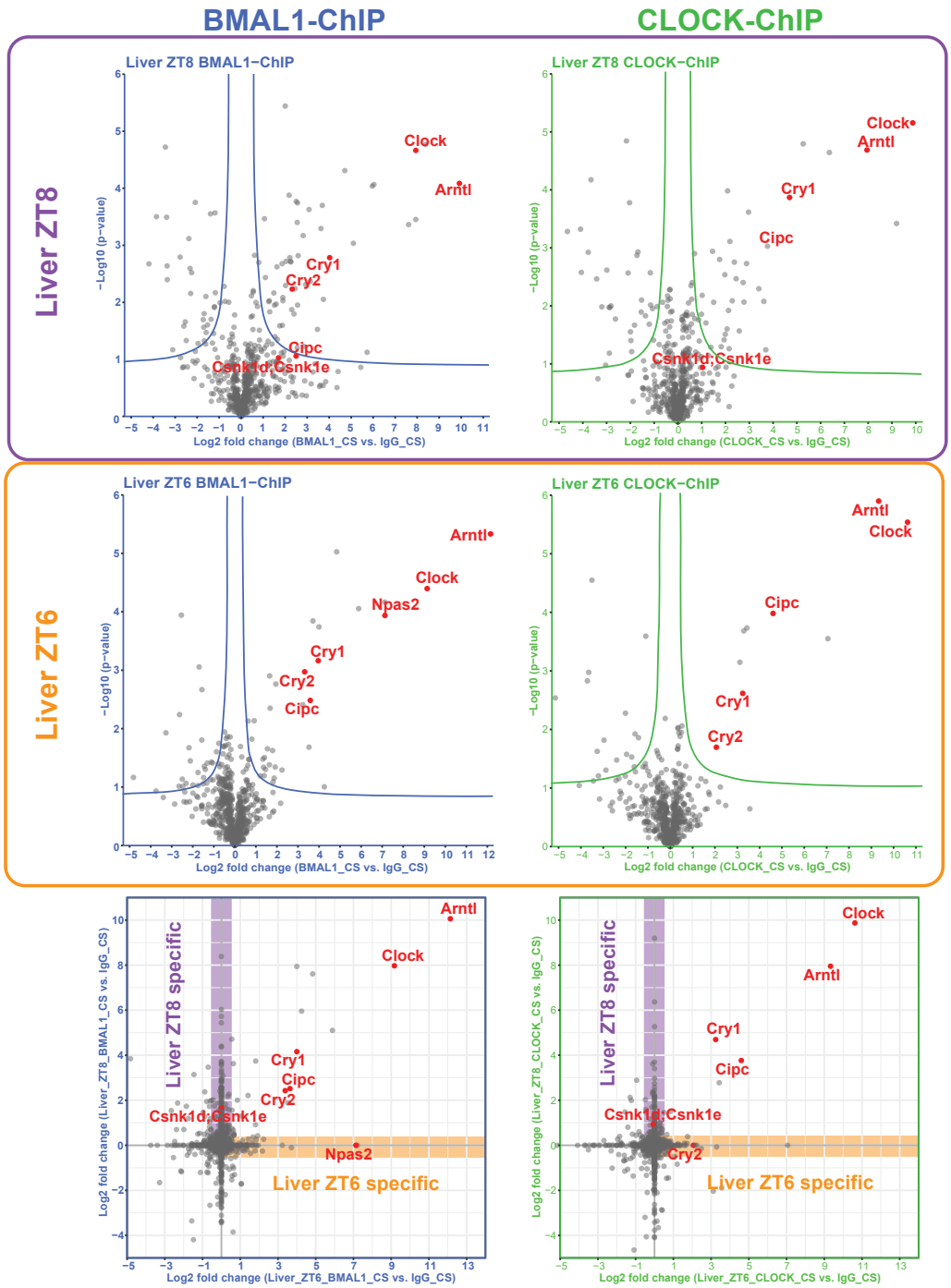

Fig. 11. ChIP-MS at different time points in mouse liver. Volcano plots illustrating the Log2 fold change and -Log10 p-value using the antibodies targeting BMAL1 or CLOCK over the IgG control in mouse liver harvested at ZT8-9 (purple box) and at ZT6 (orange box). Scatter plots on the bottom showing Log2 fold changes from the BMAL1-ChIPs (left) and CLOCK-ChIPs (right) plotted against each other. Known clock components are labeled in red.

I next tested whether the ChIP-MS method can also be applied in other tissues to successfully immunoprecipitate BMAL1:CLOCK complexes and eventually find tissue specific interactors. For this reason, I performed ChIP-MS in kidney from the same mouse I obtained the liver from at ZT6. The results demonstrated that the protocol as well as the CLOCK

and BMAL1 antibody can be used in mouse kidney as well. The bait proteins were the most significant outliers compared to the IgG control and other clock proteins were successfully immunoprecipitated, similar to what I found in liver at the same time point (Fig. 12). The clock proteins in complex with BMAL1 are comparable in liver and kidney at ZT6. In contrast, in the CLOCK-ChIPs in kidney NPAS2 was now detected whereas CRYs were not significantly enriched. The lack of significant binding of CRYs to CLOCK in kidney and the absence of NPAS2 in CLOCK complexes in liver could be due to stoichiometric effects or the proteins were really absent.

A tissue-specific complex can be further delineated by defining those proteins exclusively enriched in one of the tissues. For example, exclusively in kidney, GPX3 was immunoprecipitated by BMAL1 as well as CLOCK (scatter plots in Fig. 12, labeled in black). GPX3 is a gluathione peroxidase which is mainly expressed in kidney and then secreted into the blood. However, there are studies reporting the presence of GPX3 in the cell nucleus [126, 127]. GPX3, as all members of the gluathione peroxidase family, is envolved in redox sensing and regulation. The function of the gluathione peroxidase family members does not seem to be redundant, since they also fullfill individual roles in different pathways. However, the exact function of GPX3 is still under debate [126, 128]. Further studies are necessary to functionally validate the interaction of GPX3 with BMAL1:CLOCK in mouse kidney and its potential role as a switch to temporally regulate redox signals in this tissue.

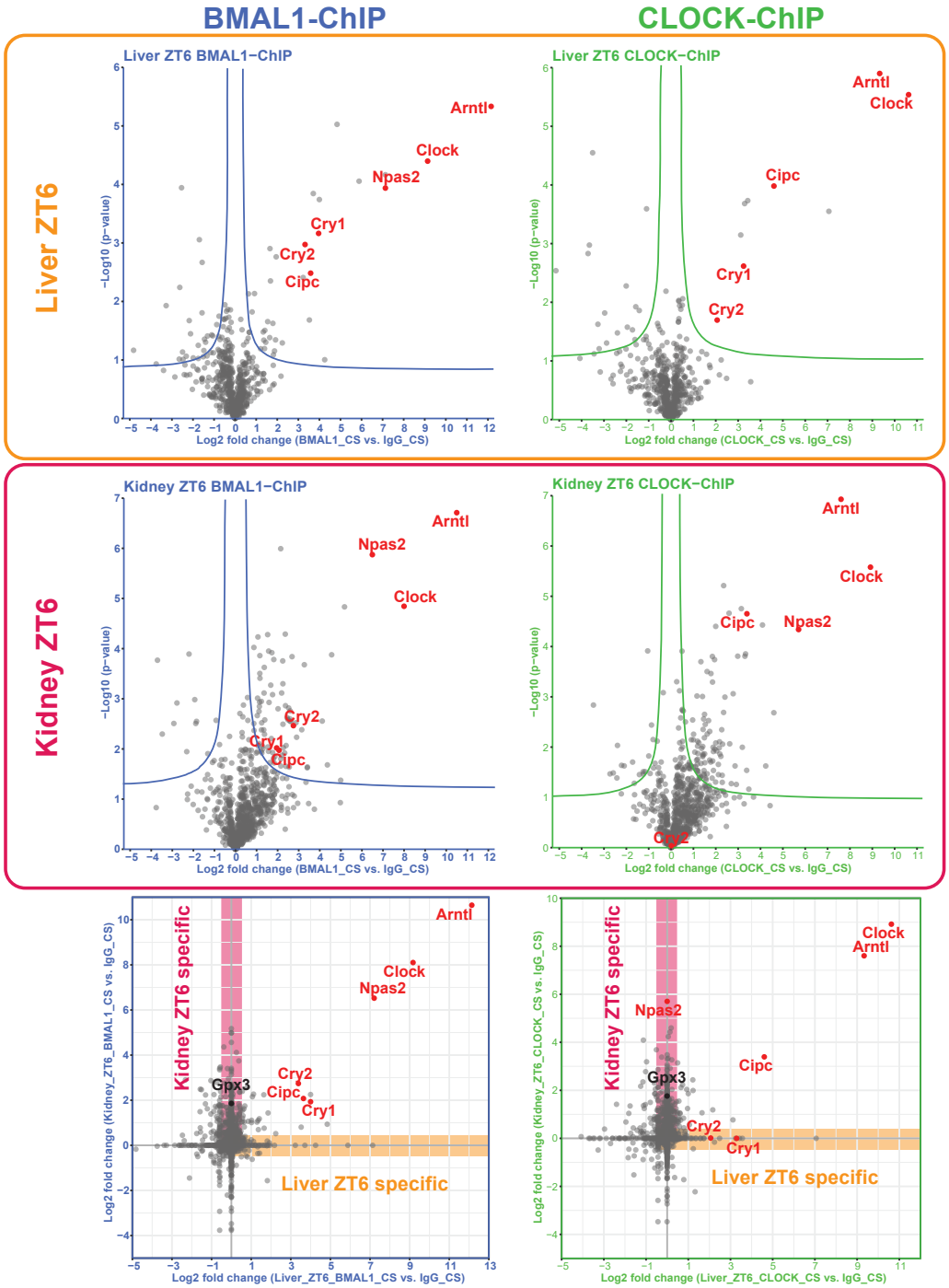

Fig. 12. ChIP-MS in mouse liver and kidney at ZT6. Volcano plots illustrating the Log2 fold change and -Log10 p-value using the antibodies targeting BMAL1 or CLOCK over the IgG control in mouse liver (orange box) and kidney (pink box). Scatter plots on the bottom showing Log2 fold changes from the BMAL1-ChIPs (left) and CLOCK-ChIPs (right) plotted against each other. Known clock components are labeled in red.

Collectively, all of the extensive preliminary results of the ChIP-MS optimization in different tissues and cell extracts indicate that this method is a very promising and effective tool to study tissue-specific and time-dependent interactions of the BMAL1 and CLOCK core clock complexes and potentially of other alternative complexes.

2.2 Multiomics Study linking Sleep to synaptic Function

2.2.1 Project Aim and Summary

Sleep is a fundamental process for consolidation of learning and memory. Insufficient amount of sleep is associated with cognitive impairment, reversible by sleep [129]. Our current knowledge indicates that sleep is regulated by two processes: the internal timing system (circadian clock) and the homeostatic process (sleep pressure) [39]. Both processes are tightly connected but it is unclear how they molecularly interact to regulate sleep. Sleep is thought to prevent synapses from becoming oversaturated during wakefulness. Thus, synapses as the basis for neuronal plasticity would be an important target for the sleep function [43]. We were intrigued by the idea of dissecting the influence of the circadian clock and the sleep homeostat on synaptic function. To do that we performed a multiomics study to molecularly investigate the influence of sleep on potential daily oscillations of mRNA, protein and phosphorylation abundances.

First, we needed to ascertain whether there are detectable oscillations at all of those mentioned levels in synapses. That is why we isolated across the day synapses from mouse forebrain containing cortex and hippocampus, brain areas with important cognitive functions such as learning [42]. The synaptic fractions, synaptoneurosomes, were then analyzed using deep-sequencing and advanced MS.

The major finding at the mRNA level was that about two-thirds of synaptic transcripts showed daily oscillations in abundance with the majority of transcripts peaking preceding dawn and a less pronounced cluster anticipating dusk. Transcripts in both cluster coded proteins with distinct functions, metabolism and translation preceding dawn, and synaptic translation anticipating dusk. Our parallel in-depth proteome revealed that 75% of the rhythmic proteins peaked at the time of their corresponding oscillating transcript implying local synaptic translation. At the post-translational level, we found that 30% of the more than 7000 quantified phosphopeptides in synapses showed daily changes in their abundance and those rhythms were almost independent of the oscillations of the proteome, suggesting that phosphorylation imposes another layer of regulation at the synapses. The distribution of the phases of these oscillating phosphopeptides separated into two major clusters, corresponding to dawn and dusk or in other words to the transition from wake to sleep and vise versa.

Among the quantified cycling phosphopeptides, a variety originated from kinases from all major kinase families indicating that phosphorylation-dependent activation of the kinases in response to rest-activity cycles is a key driver to regulate synaptic homeostasis and function. However, because site-specific phosphorlyation cannot be directly linked to kinase activity, we applied the PHOTON pipeline [130], a method to predict the time of day activation of proteins in their signaling context, resulting in activation patterns for some of the cycling

phosphorylated kinases.
Second, we wanted to identify whether the oscillations are driven by the circadian clock or by the sleep homeostat. To do that, we applied our multiomics study to isolated synapses from mice, which were sleep deprived 4h before being sacrificed at different times across the day; this procedure allows to have a similarly increased sleep pressure at all time points. Notably, while sleep deprivation abolished the rhythms of proteins at the synapses, transcript cycles remained nearly unaffected. This indicates that the circadian clock provides the synapses with transcripts before dawn and dusk, which will be translated in response to rest-activity. At the post-translational level, 98% of the rhythmic phosphorylation were lost due to sleep deprivation. Only phosphorylations in 41 proteins remained rhythmic suggesting that the circadian clock may regulate the function of those proteins in forebrain synaptoneurosomes. Together, our data provide the evidence that sleep need and molecular signatures of synaptic function are tightly linked. Moreover, we offer the first comprehensive overview of global changes in mRNA, protein and phosphoprotein abundances in mouse synapses across a day. Summarizing, our data indicate that in synapses the circadian clock is the main driver of cycles of mRNA levels while oscillations of protein and phosphorylation levels are driven by sleep-wake cycles.

2.2.2 Contribution and Publications

The project was done in collaboration with Steve Brown's Lab at the University of Zurich and was published in two back-to-back research articles in Science.

The forebrain synaptic transcriptome is organized by clocks but its proteome is driven by sleep

Sara B. Noya, David Colameo, Franziska Brüning, Andrea Spinnler, Dennis Mircsof, Lennart Opitz, Matthias Mann, Shiva K. Tyagarajan, Maria S. Robles, Steven A. Brown
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Sleep-wake cycles drive daily dynamics of synaptic phosphorylation

Franziska Brüning*, Sara B. Noya*, Tanja Bange, Stella Koutsouli, Jan D. Rudolph, Shiva K. Tyagarajan, Jürgen Cox, Matthias Mann, Steven A. Brown, Maria S. Robles
Science. 2019 October; 366(6462)

I performed the sample preparation, the data acquisition and analysis for the proteome and phosphoproteome samples under the supervision of Maria Robles and in collaboration with Sara Noya and Steve Brown. In addition, I prepared, together with Sara Noya the article figures and wrote, together with Maria Robles, Sara Noya and Steve Brown the manuscript of the phosphorylation study.

3 Conclusion and Outlook

The aim of one of my PhD projects was to set up a method to molecularly study endogenous CLOCK and BMAL1 complexes. The transcription factors CLOCK and BMAL1, as core clock components, are known to form a complex protein network, that changes over time in the promoters of clock controlled genes to facilitate their rhythmic expression. Despite the identification of several proteins, binding to core clock components, the interaction network of the molecular clock complex, that precisely times important physiological processes in the cell and throughout the body, remains incomplete.
I successfully optimized a chromatin immunoprecipitation protocol followed by advanced mass spectrometry to purify and identify components of endogenous protein complexes bound to chromatin, containing CLOCK and BMAL1, in human cell lines and murine tissue. This method can now be used in the laboratory to obtain further insights on the molecular mechanism of the clock by analyzing the BMAL1:CLOCK complex compositions in, first, a time-dependent and, second, in a tissue-dependent manner, in both cells and tissues. For instance, with dexamethasone synchronized U-2 OS cells and tissues from entrained mice this method can be used to explore temporal changes in the protein composition of the core clock complex and to thus identify time dependent novel interactors. The combination of specific antibodies with different control conditions, such as knock-out cell lines/tissues of the bait as well as IgG controls could not only allow us to find novel complex constituents but also to investigate their stoichiometry.
Furthermore, performing ChIP-MS in different tissues to study the interactions of BMAL1: CLOCK with other proteins might be especially interesting because it is highly debated how the same heterodimer complex can drive tissue-specific metabolism throughout the body. Thus, the comparison of complex constituents obtained in different tissues will allow us to identify tissue-specific regulators responsible for organ-specific transcriptional outputs. Moreover, future experiments could be performed with the aim to construct a "circadian core clock protein-interaction atlas in different tissue" by performing ChIP-MS in a tissue- and time-dependent manner using various tissue harvested at different times of the day.
Additionally, the ChIP-MS protocol can also be performed to study the negative loop if suitable antibodies are available detecting the core repressors, CRYs and PERs. In general, the ChIP-MS method is not restricted to the circadian field but rather can be applied to study DNA-protein complexes in static but also kinetic manners in any other biological field.

The multiomics study of total mouse forebrains and isolated synapses, presented in this thesis, provides a valuable molecular resource for the circadian and the sleep field. We detected oscillations of transcript, protein and phosphorylation abundances across the day with patterns and degrees that greatly differed between total forebrain and the synaptic

compartment. This indicated that the combination of temporal and spatial omics provides a much deeper and comprehensive picture of dynamic and local biological functions. By combining a sleep deprivation protocol to this innovative multiomics approach, we investigated how circadian and sleep-wake cycles shaped synaptic omics dynamics. We thereby found that the main driver of mRNA oscillations is the circadian clock while sleep-wake cycles are responsible for the shapping of protein and phosphorlyation rhythms. Thus, our study revealed that the synaptic processes in the forebrain are regulated by the circadian clock as well as the sleep homeostatic process and can therefore be considered as an ideal neuronal compartment for future studies trying to unravel the detailed mechanism of the sleep.
Our findings highlight the importance to not only perform studies on the organ level but also on subcellular compartments since, for example, the rhythmic synaptic phosphoproteome greatly differed from the oscillations when using total forebrain. Hence, future proteomics studies in other subcellular neuronal compartments, such as nuclei, may shed light on how sleep is molecularly regulated at the cellular level and how sleep deprivation impacts diverse cellular processes, such as transcription.
Together, since almost all synaptic phosphorylations were greatly influenced by sleep, our data contain proteins which phosphorylation status could be used as potential markers of sleep or wake need, something that is still under debate in the sleep field. Interestingly, among the over 2000 cycling phosphopeptides many of them belonged to kinases themselves. The majority was peaking at the transition from sleep to wake, suggesting that sleep-wake cycles drive kinase function in synapses which in turn drives downstram phosphorylation dependent signaling pathways. Indeed, our study uncovered a large network of kinase substrates with phosphorylations temporally driven by sleep-wake which could be considered as the molecular core of sleep-driven synaptic function, which is still one of the major unanswered scientific questions in the sleep field. Future functional studies could allow to obtain detailed information about the precise role of those uncovered kinases in the molecular control of sleep-wake cycles.

In sum, my two PhD projects make a fundamental contribution to the understanding of pivotal open questions in circadian biology and sleep. In addition, the established ChIP-MS method, as a useful tool for the laboratory, will allow to answer further questions regarding how the CLOCK and BMAL1 complex composition is dynamically established in a time- and tissue-specific manner, paving the way for additional long term projects.

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